FIG.1

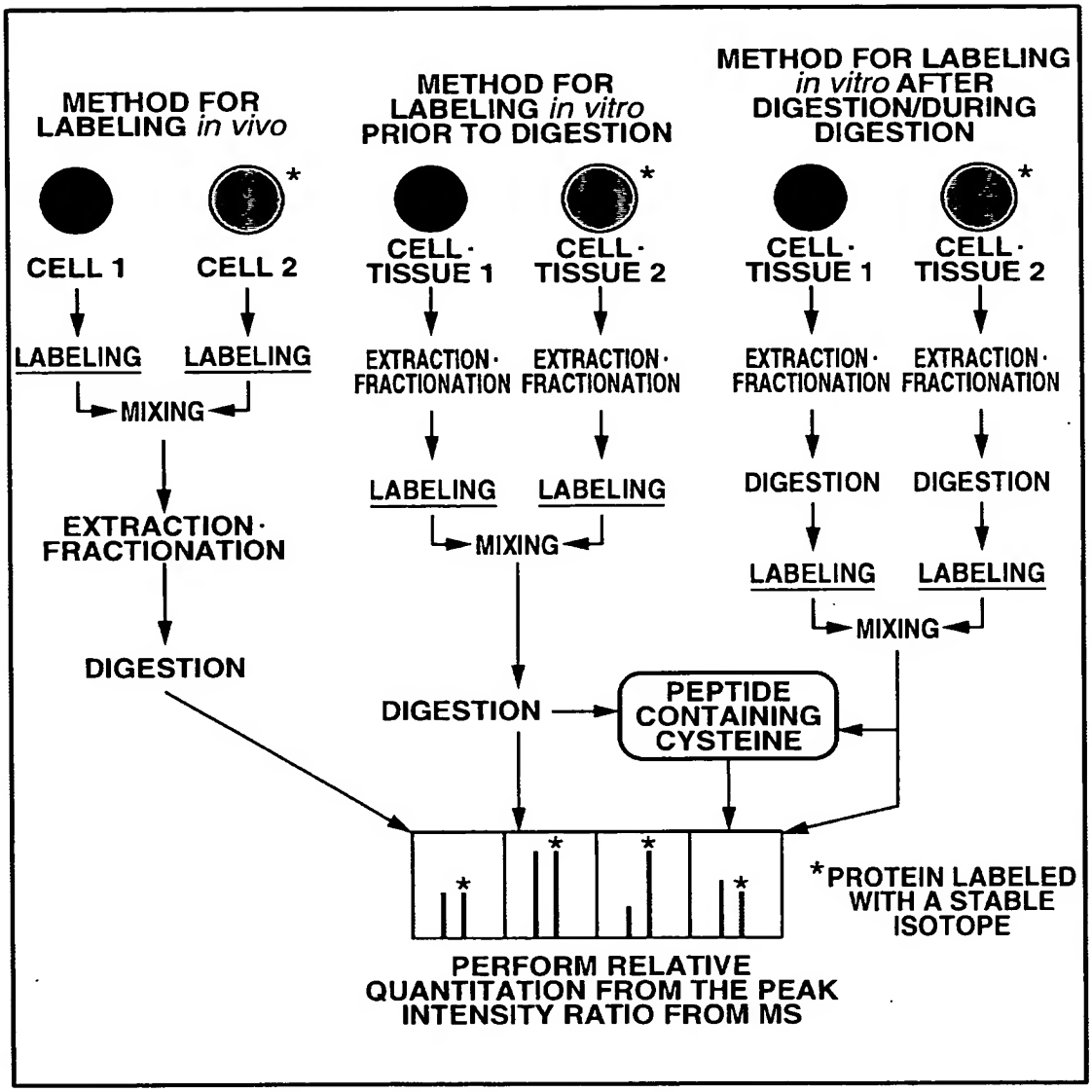
METHOD FOR LABELING *in vivo*
METHOD FOR LABELING *in vitro* PRIOR TO DIGESTION
METHOD FOR LABELING *in vitro* AFTER DIGESTION/DURING DIGESTION
CELL 1
CELL 2
CELL · TISSUE 1
CELL · TISSUE 2
CELL · TISSUE 1
CELL · TISSUE 2
LABELING
LABELING
EXTRACTION · FRACTIONATION
EXTRACTION · FRACTIONATION
EXTRACTION · FRACTIONATION
EXTRACTION · FRACTIONATION
MIXING
LABELING
LABELING
DIGESTION
DIGESTION
MIXING
EXTRACTION · FRACTIONATION
LABELING
LABELING
DIGESTION
MIXING
DIGESTION
PEPTIDE CONTAINING CYSTEINE
*PROTEIN LABELED WITH A STABLE ISOTOPE
PERFORM RELATIVE QUANTITATION FROM THE PEAK INTENSITY RATIO FROM MS

FIG.2

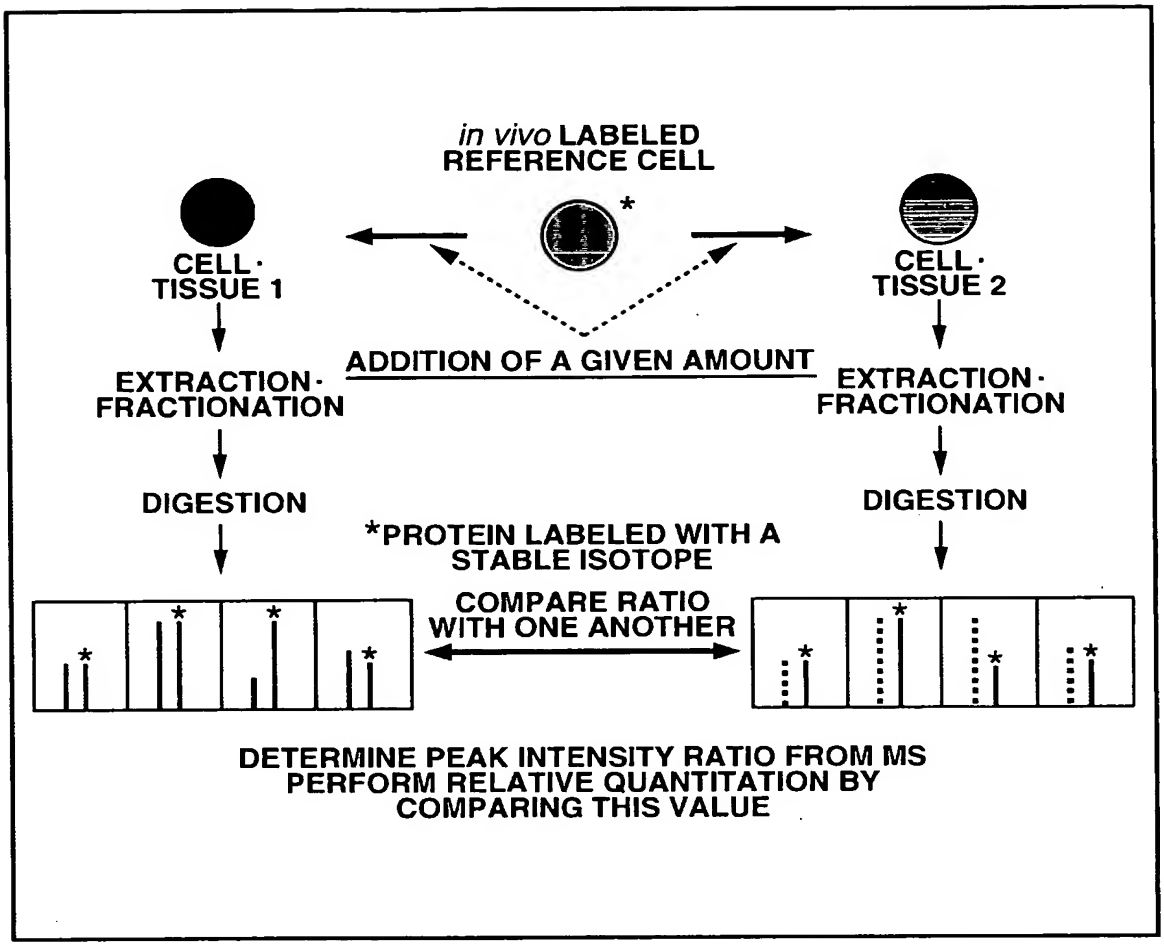
in vivo LABELED REFERENCE CELL
CELL · TISSUE 1
CELL · TISSUE 2
ADDITION OF A GIVEN AMOUNT
EXTRACTION · FRACTIONATION
EXTRACTION · FRACTIONATION
DIGESTION
DIGESTION
*PROTEIN LABELED WITH A STABLE ISOTOPE
COMPARE RATIO WITH ONE ANOTHER
DETERMINE PEAK INTENSITY RATIO FROM MS
PERFORM RELATIVE QUANTITATION BY
COMPARING THIS VALUE

FIG.3

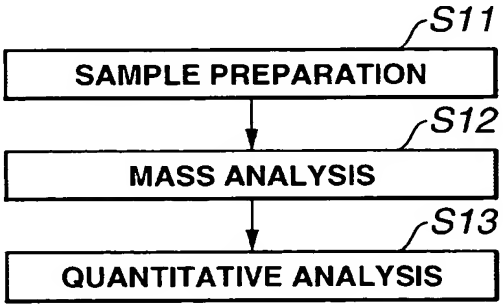
S11
SAMPLE PREPARATION
S12
MASS ANALYSIS
S13
QUANTITATIVE ANALYSIS

FIG.4

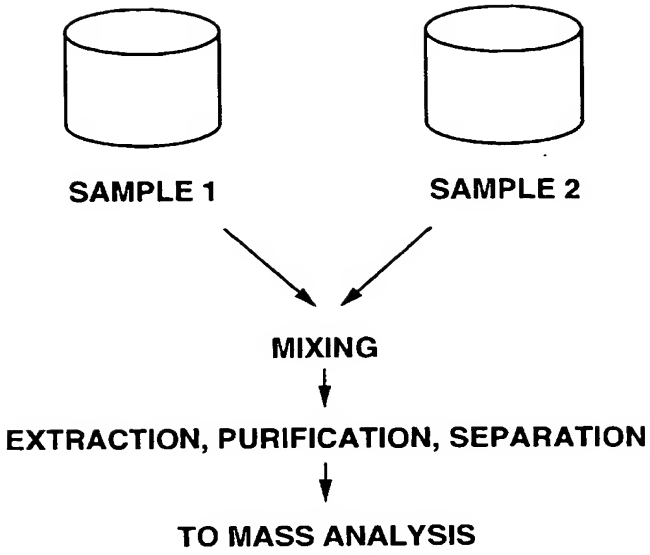
SAMPLE 1
SAMPLE 2
MIXING
EXTRACTION, PURIFICATION, SEPARATION
TO MASS ANALYSIS

FIG.5

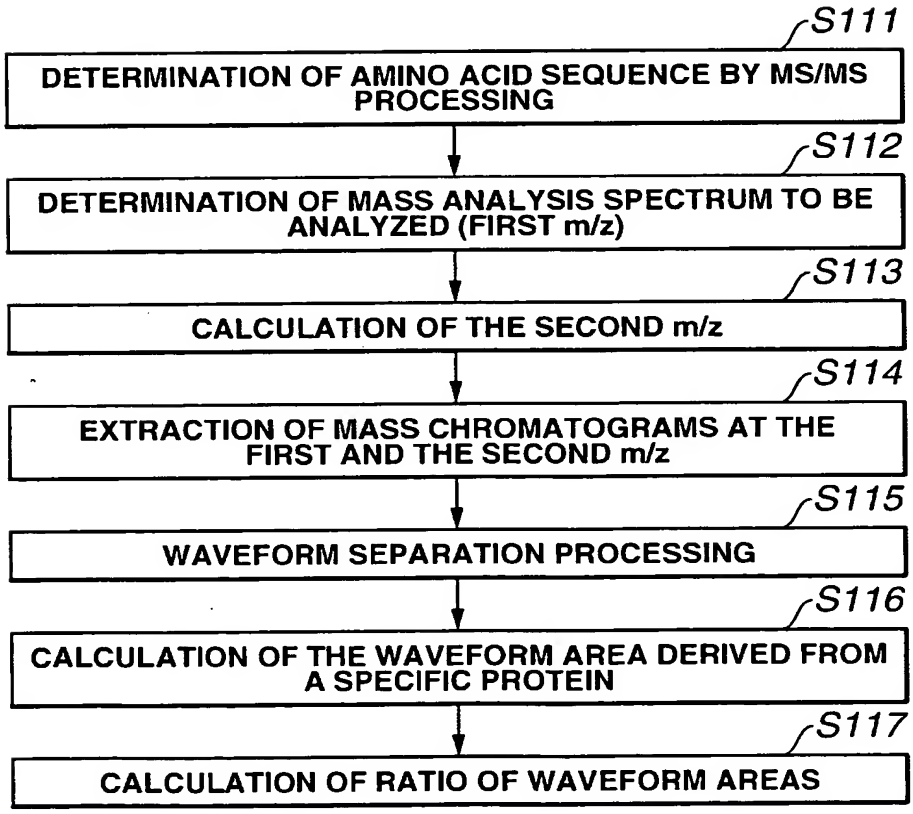
S111
DETERMINATION OF AMINO ACID SEQUENCE BY MS/MS PROCESSING
S112
DETERMINATION OF MASS ANALYSIS SPECTRUM TO BE ANALYZED (FIRST m/z)
S113
CALCULATION OF THE SECOND m/z
S114
EXTRACTION OF MASS CHROMATOGRAMS AT THE FIRST AND THE SECOND m/z
S115
WAVEFORM SEPARATION PROCESSING
S116
CALCULATION OF THE WAVEFORM AREA DERIVED FROM A SPECIFIC PROTEIN
S117
CALCULATION OF RATIO OF WAVEFORM AREAS

FIG.6

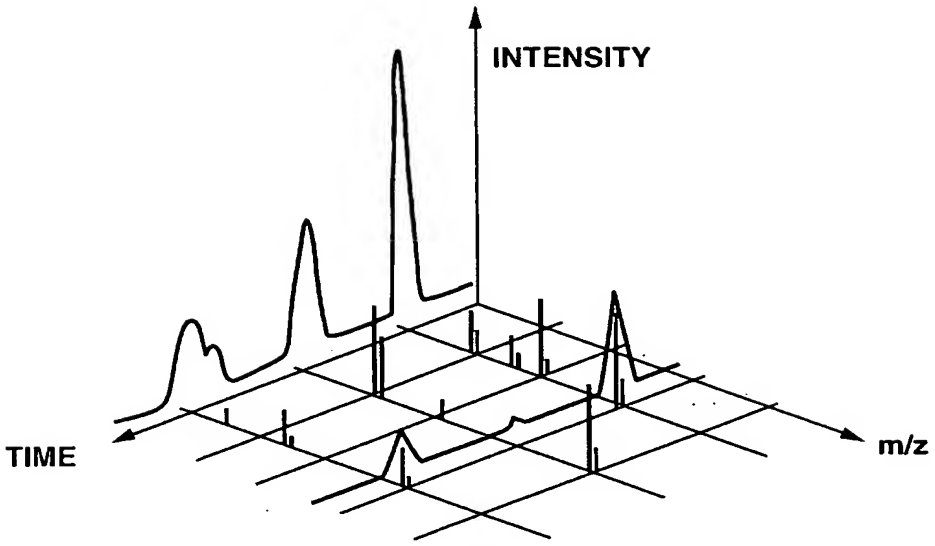

FIG.7

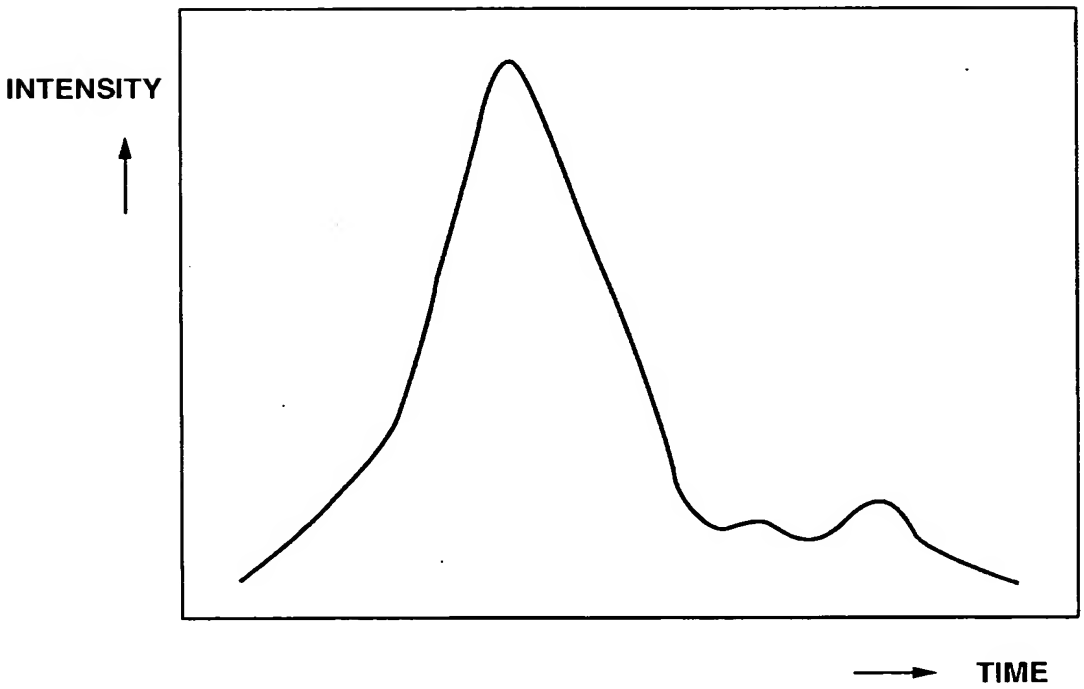

FIG.8

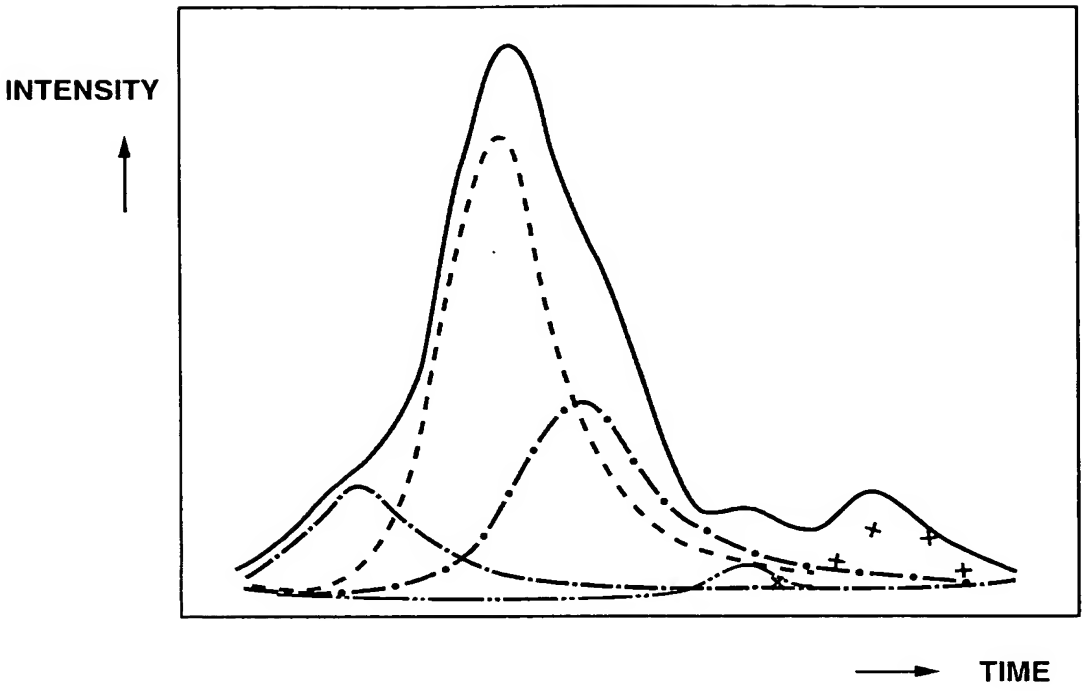

FIG.9

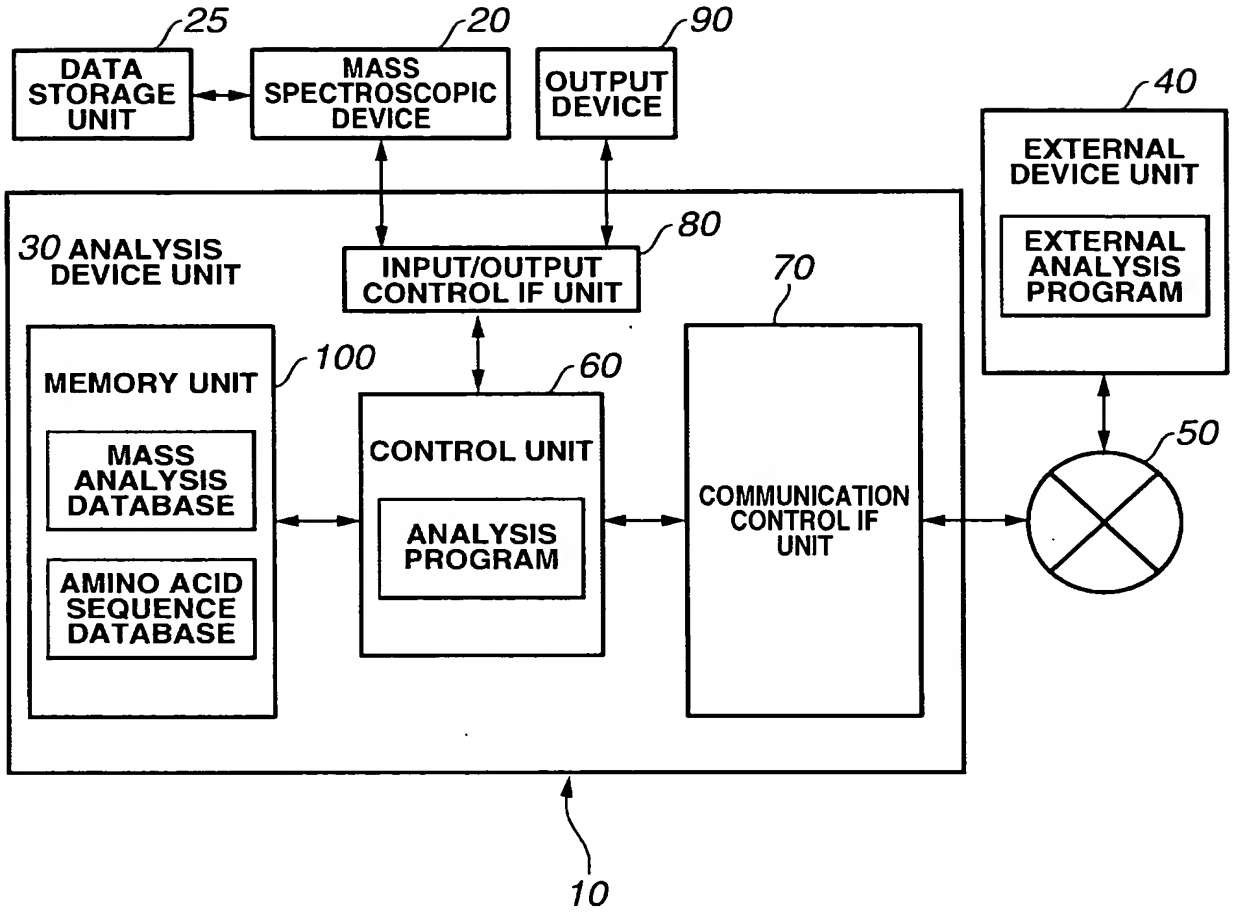
25
DATA STORAGE UNIT
20
MASS SPECTROSCOPIC DEVICE
90
OUTPUT DEVICE
40
EXTERNAL DEVICE UNIT
EXTERNAL ANALYSIS PROGRAM
30 ANALYSIS DEVICE UNIT
80
INPUT/OUTPUT CONTROL IF UNIT
70
MEMORY UNIT
100
60
CONTROL UNIT
MASS ANALYSIS DATABASE
ANALYSIS PROGRAM
50
COMMUNICATION CONTROL IF UNIT
AMINO ACID SEQUENCE DATABASE
10

FIG.10

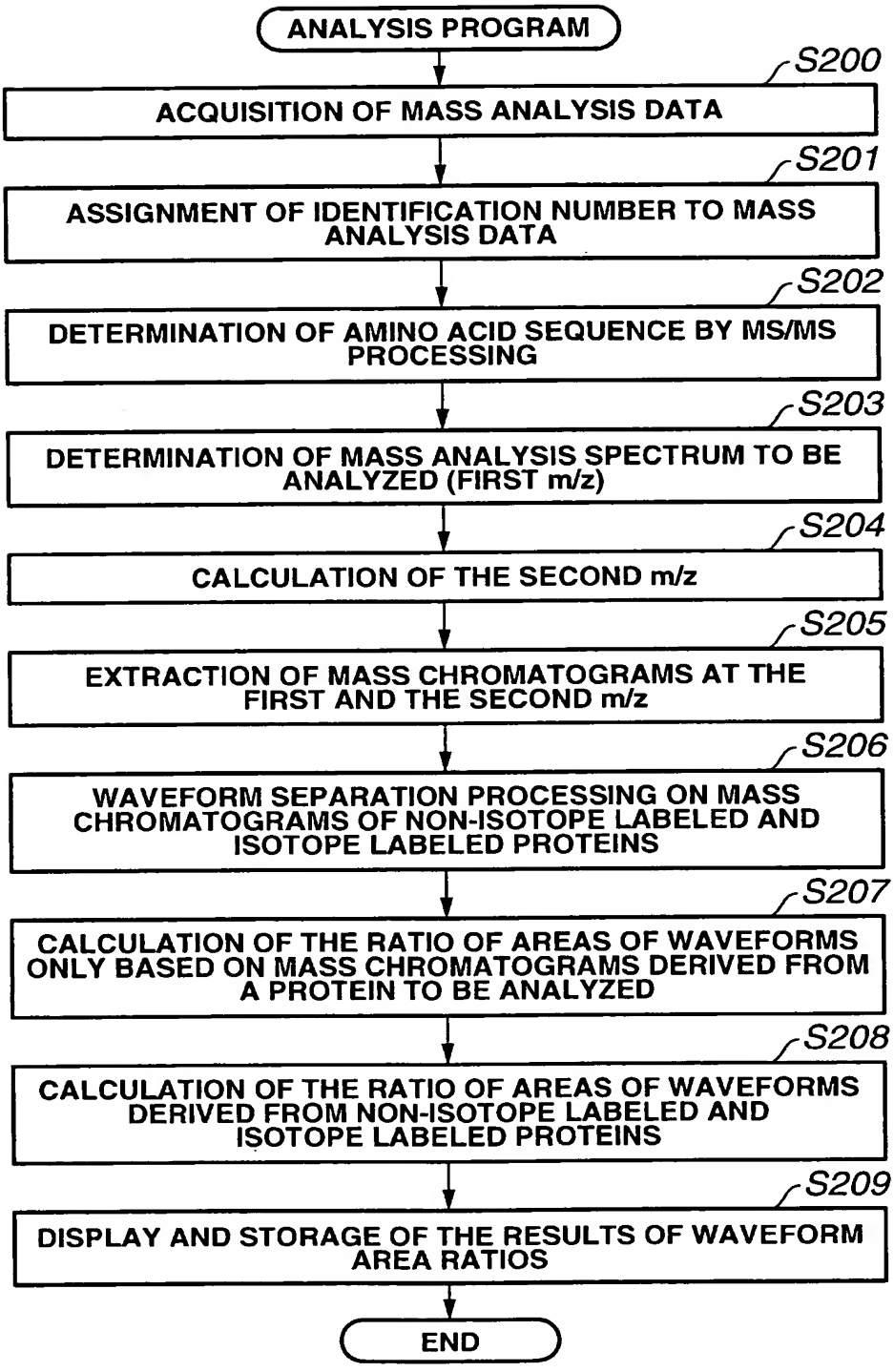
ANALYSIS PROGRAM
S200
ACQUISITION OF MASS ANALYSIS DATA
S201
ASSIGNMENT OF IDENTIFICATION NUMBER TO MASS ANALYSIS DATA
S202
DETERMINATION OF AMINO ACID SEQUENCE BY MS/MS PROCESSING
S203
DETERMINATION OF MASS ANALYSIS SPECTRUM TO BE ANALYZED (FIRST m/z)
S204
CALCULATION OF THE SECOND m/z
S205
EXTRACTION OF MASS CHROMATOGRAMS AT THE FIRST AND THE SECOND m/z
S206
WAVEFORM SEPARATION PROCESSING ON MASS CHROMATOGRAMS OF NON-ISOTOPE LABELED AND ISOTOPE LABELED PROTEINS
S207
CALCULATION OF THE RATIO OF AREAS OF WAVEFORMS ONLY BASED ON MASS CHROMATOGRAMS DERIVED FROM A PROTEIN TO BE ANALYZED
S208
CALCULATION OF THE RATIO OF AREAS OF WAVEFORMS DERIVED FROM NON-ISOTOPE LABELED AND ISOTOPE LABELED PROTEINS
S209
DISPLAY AND STORAGE OF THE RESULTS OF WAVEFORM AREA RATIOS
END

FIG.11

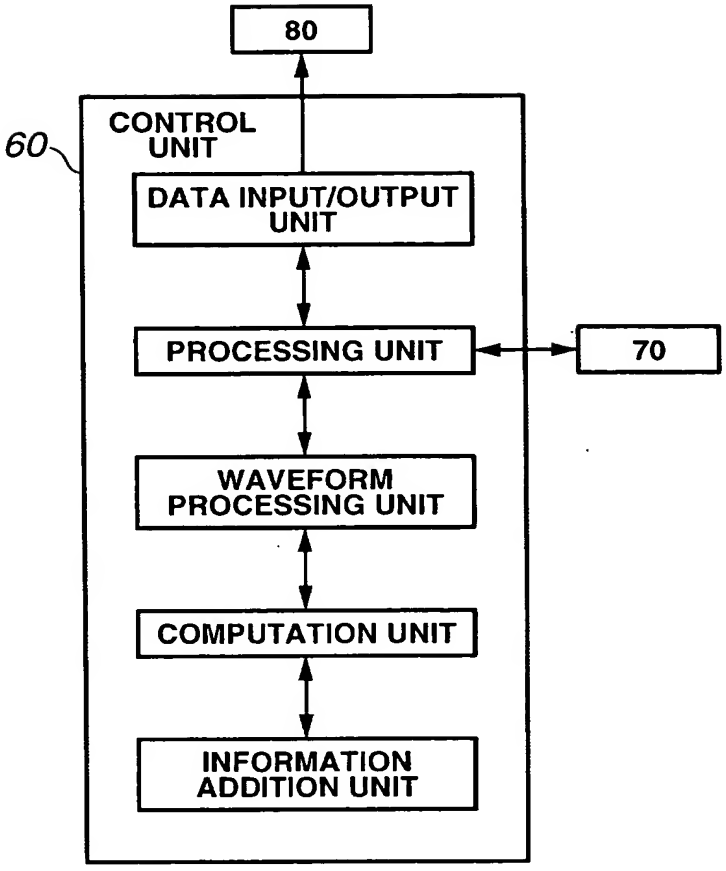
80
60
CONTROL UNIT
DATA INPUT/OUTPUT UNIT
PROCESSING UNIT
70
WAVEFORM PROCESSING UNIT
COMPUTATION UNIT
INFORMATION ADDITION UNIT

FIG.12

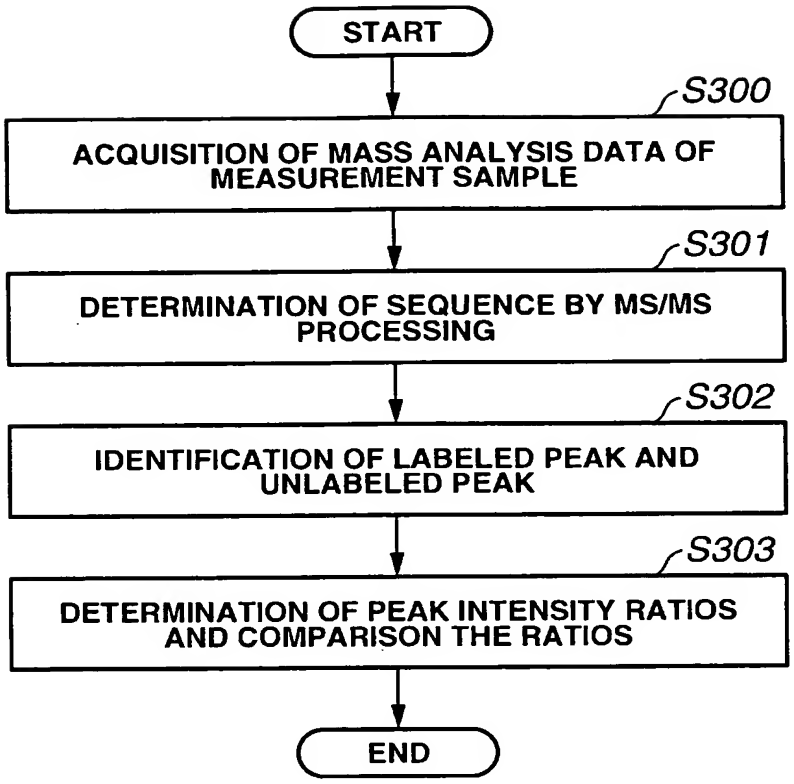
START
S300
ACQUISITION OF MASS ANALYSIS DATA OF MEASUREMENT SAMPLE
S301
DETERMINATION OF SEQUENCE BY MS/MS PROCESSING
S302
IDENTIFICATION OF LABELED PEAK AND UNLABELED PEAK
S303
DETERMINATION OF PEAK INTENSITY RATIOS AND COMPARISON THE RATIOS
END

FIG.13

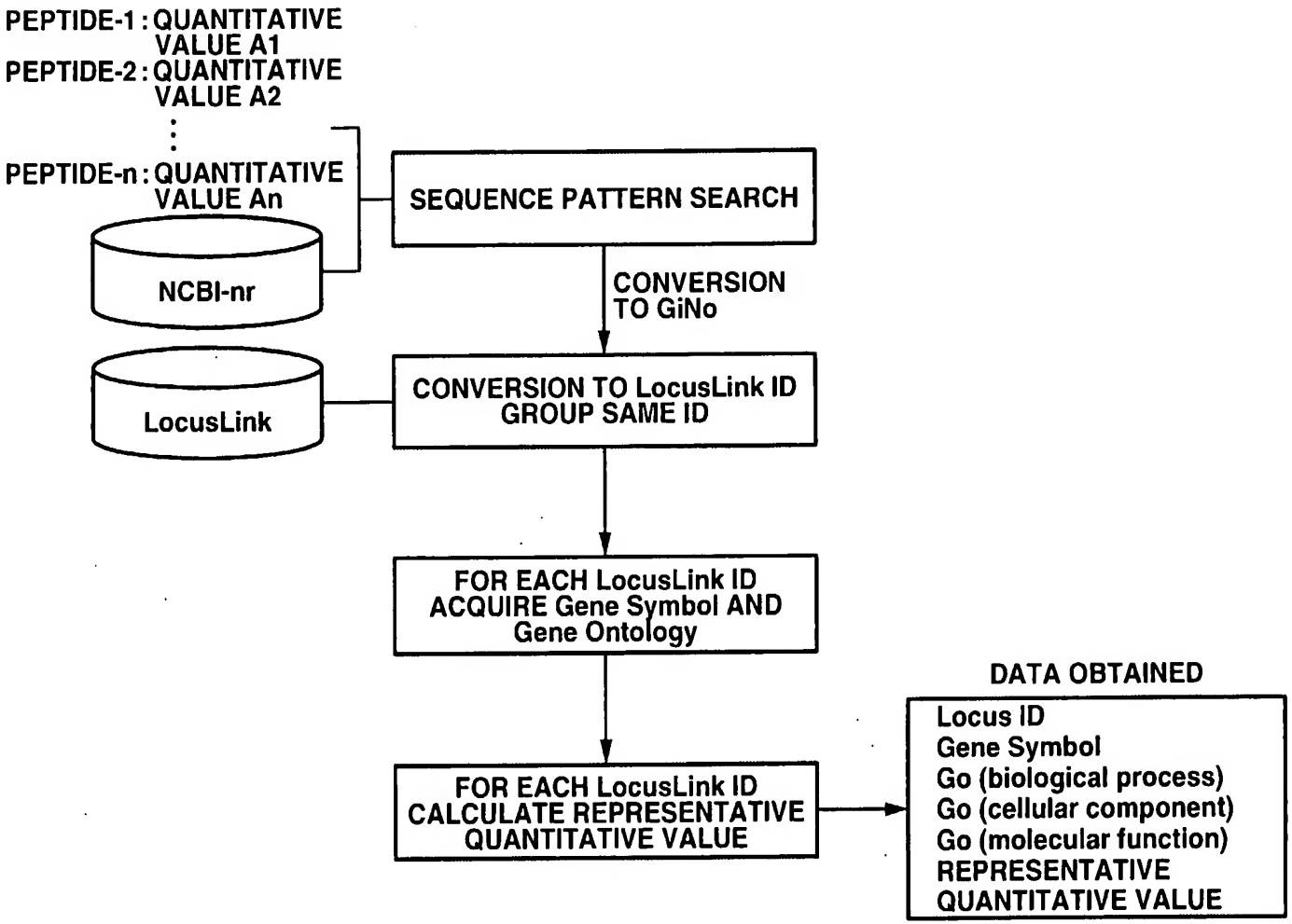
PEPTIDE-1 : QUANTITATIVE VALUE A1
PEPTIDE-2 : QUANTITATIVE VALUE A2
PEPTIDE-n : QUANTITATIVE VALUE An
NCBI-nr
LocusLink
SEQUENCE PATTERN SEARCH
CONVERSION TO GiNo
CONVERSION TO LocusLink ID GROUP SAME ID
FOR EACH LocusLink ID ACQUIRE Gene Symbol AND Gene Ontology
FOR EACH LocusLink ID CALCULATE REPRESENTATIVE QUANTITATIVE VALUE
DATA OBTAINED
Locus ID
Gene Symbol
Go (biological process)
Go (cellular component)
Go (molecular function)
REPRESENTATIVE QUANTITATIVE VALUE

FIG.14

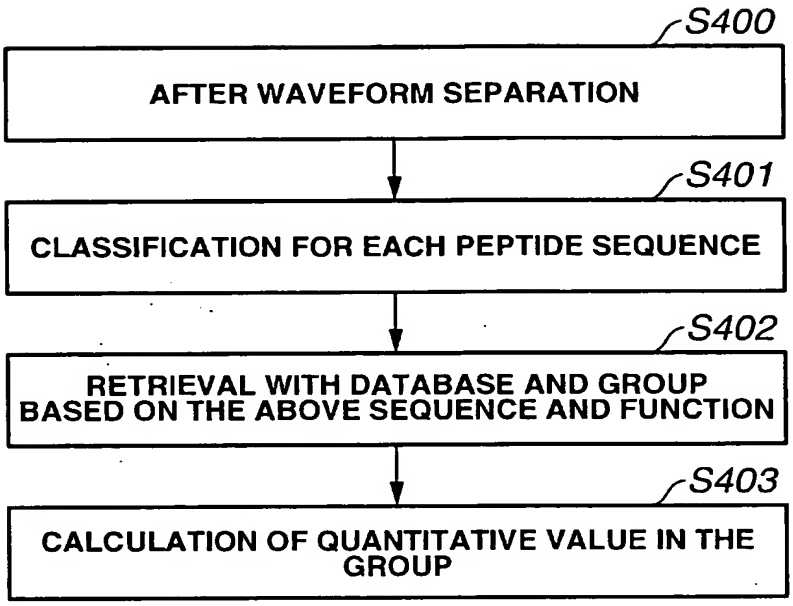
S400
AFTER WAVEFORM SEPARATION
S401
CLASSIFICATION FOR EACH PEPTIDE SEQUENCE
S402
RETRIEVAL WITH DATABASE AND GROUP BASED ON THE ABOVE SEQUENCE AND FUNCTION
S403
CALCULATION OF QUANTITATIVE VALUE IN THE GROUP

FIG.15

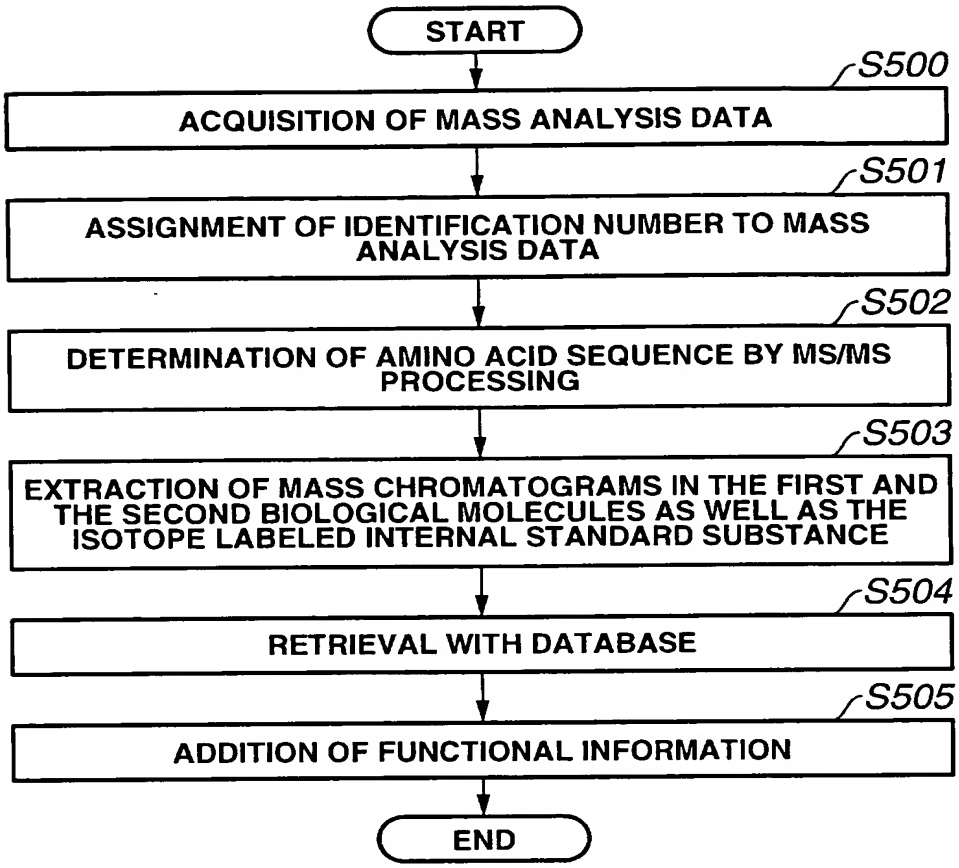
START
S500
ACQUISITION OF MASS ANALYSIS DATA
S501
ASSIGNMENT OF IDENTIFICATION NUMBER TO MASS ANALYSIS DATA
S502
DETERMINATION OF AMINO ACID SEQUENCE BY MS/MS PROCESSING
S503
EXTRACTION OF MASS CHROMATOGRAMS IN THE FIRST AND THE SECOND BIOLOGICAL MOLECULES AS WELL AS THE ISOTOPE LABELED INTERNAL STANDARD SUBSTANCE
S504
RETRIEVAL WITH DATABASE
S505
ADDITION OF FUNCTIONAL INFORMATION
END

FIG.16

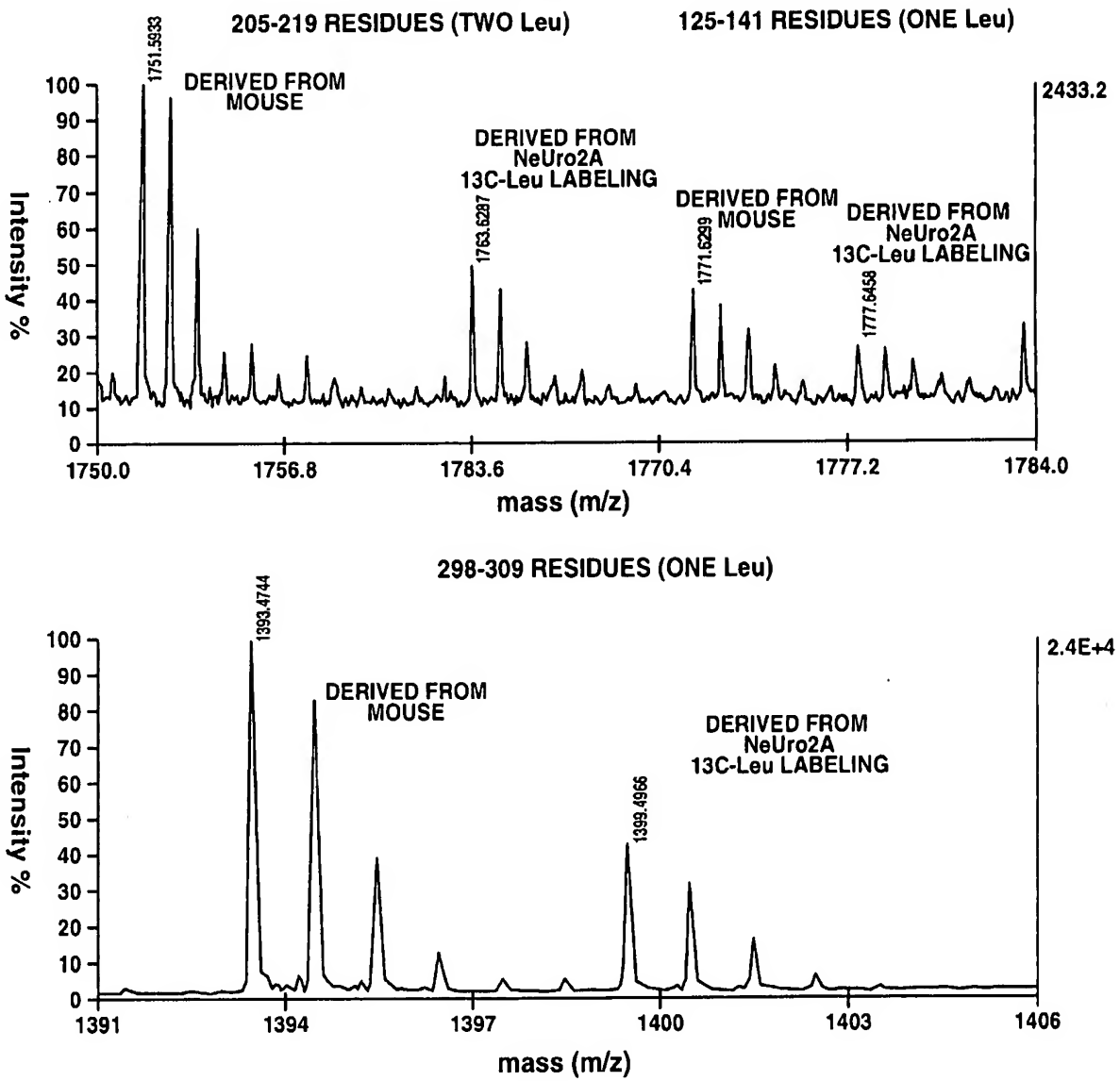
205-219 RESIDUES (TWO Leu)
125-141 RESIDUES (ONE Leu)
1751.5933
DERIVED FROM MOUSE
DERIVED FROM NeUro2A 13C-Leu LABELING
1763.6287
DERIVED FROM MOUSE
1771.6299
DERIVED FROM NeUro2A 13C-Leu LABELING
1777.6458
2433.2
Intensity %
100
90
80
70
60
50
40
30
20
10
0
1750.0
1756.8
1783.6
1770.4
1777.2
1784.0
mass (m/z)
298-309 RESIDUES (ONE Leu)
1393.4744
DERIVED FROM MOUSE
DERIVED FROM NeUro2A 13C-Leu LABELING
1399.4966
2.4E+4
Intensity %
100
90
80
70
60
50
40
30
20
10
0
1391
1394
1397
1400
1403
1406
mass (m/z)

FIG.17

REGARDING Dose Response, 298-309 RESIDUES IN cMDH
(LEFT SIDE IS DERIVED FROM MOUSE BRAIN, RIGHT SIDE IS
DERIVED FROM Neuro2A AND 13C LABELING×6 ON Leu)

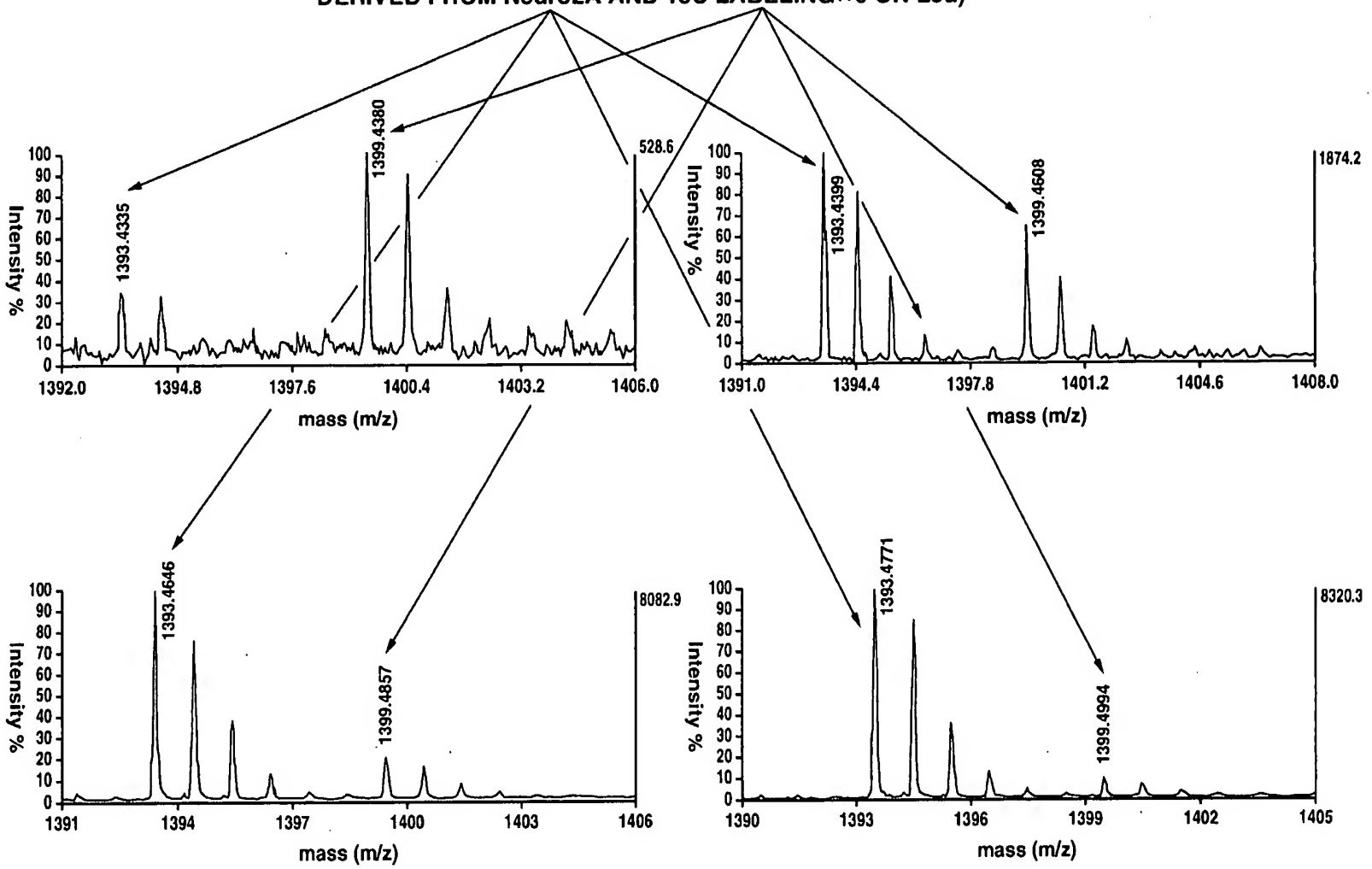

FIG.18

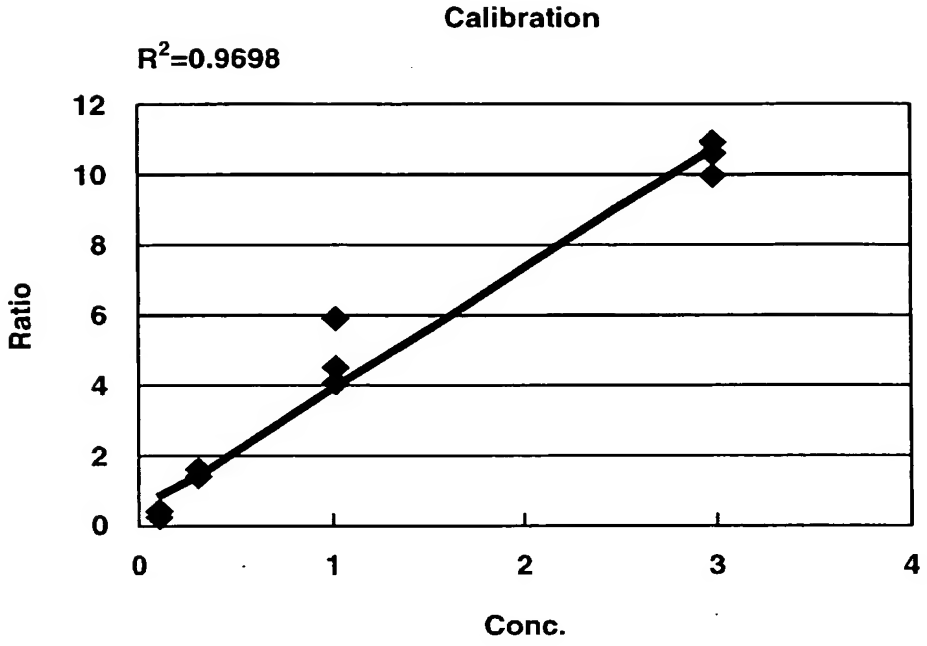

FIG.19

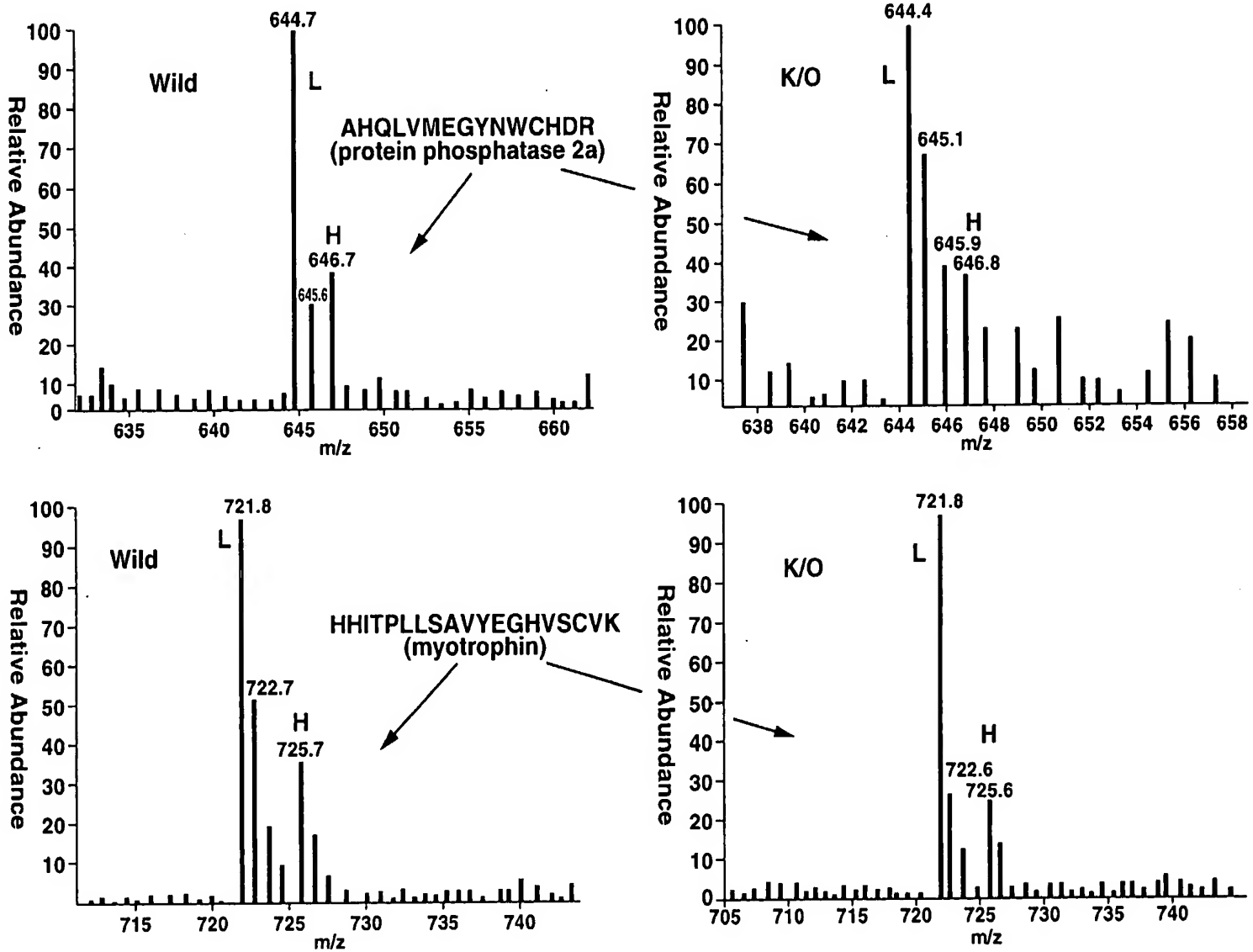
Wild
644.7
L
AHQLVMEGYNWCHDR
(protein phosphatase 2a)
H
646.7
645.6
Relative Abundance
m/z
K/O
644.4
L
645.1
H
645.9
646.8
Relative Abundance
m/z
Wild
721.8
L
HHITPLLSAVYEGHVSCVK
(myotrophin)
722.7
H
725.7
Relative Abundance
m/z
K/O
721.8
L
H
722.6
725.6
Relative Abundance
m/z

FIG.20

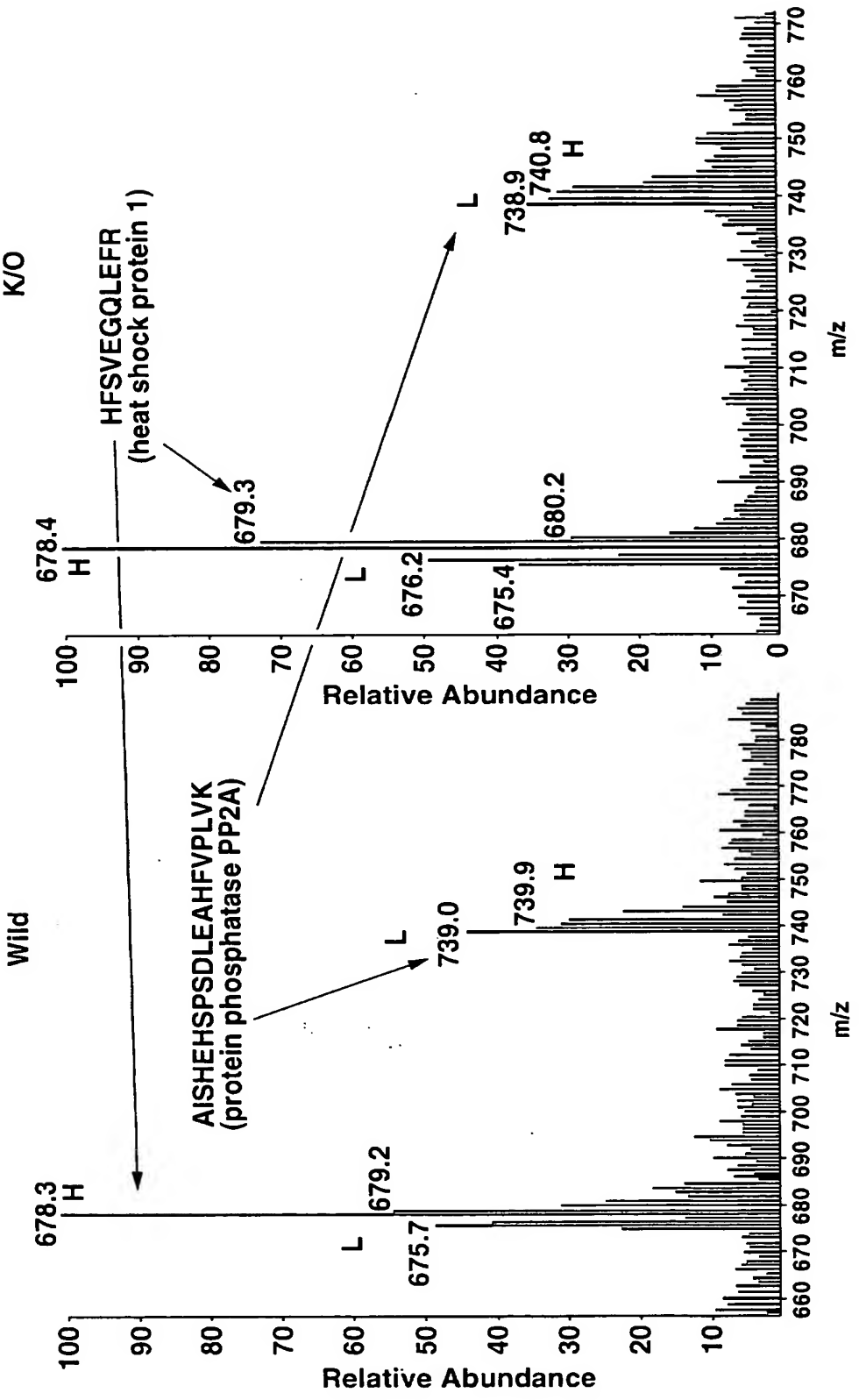
K/O
HFSVEGQLEFR
(heat shock protein 1)
678.4
H
679.3
L
676.2
675.4
680.2
L
738.9
740.8
H
Relative Abundance
m/z
Wild
AISHEHSPSDLEAHFVPLVK
(protein phosphatase PP2A)
678.3
H
679.2
L
675.7
739.0
L
739.9
H
Relative Abundance
m/z

FIG.21

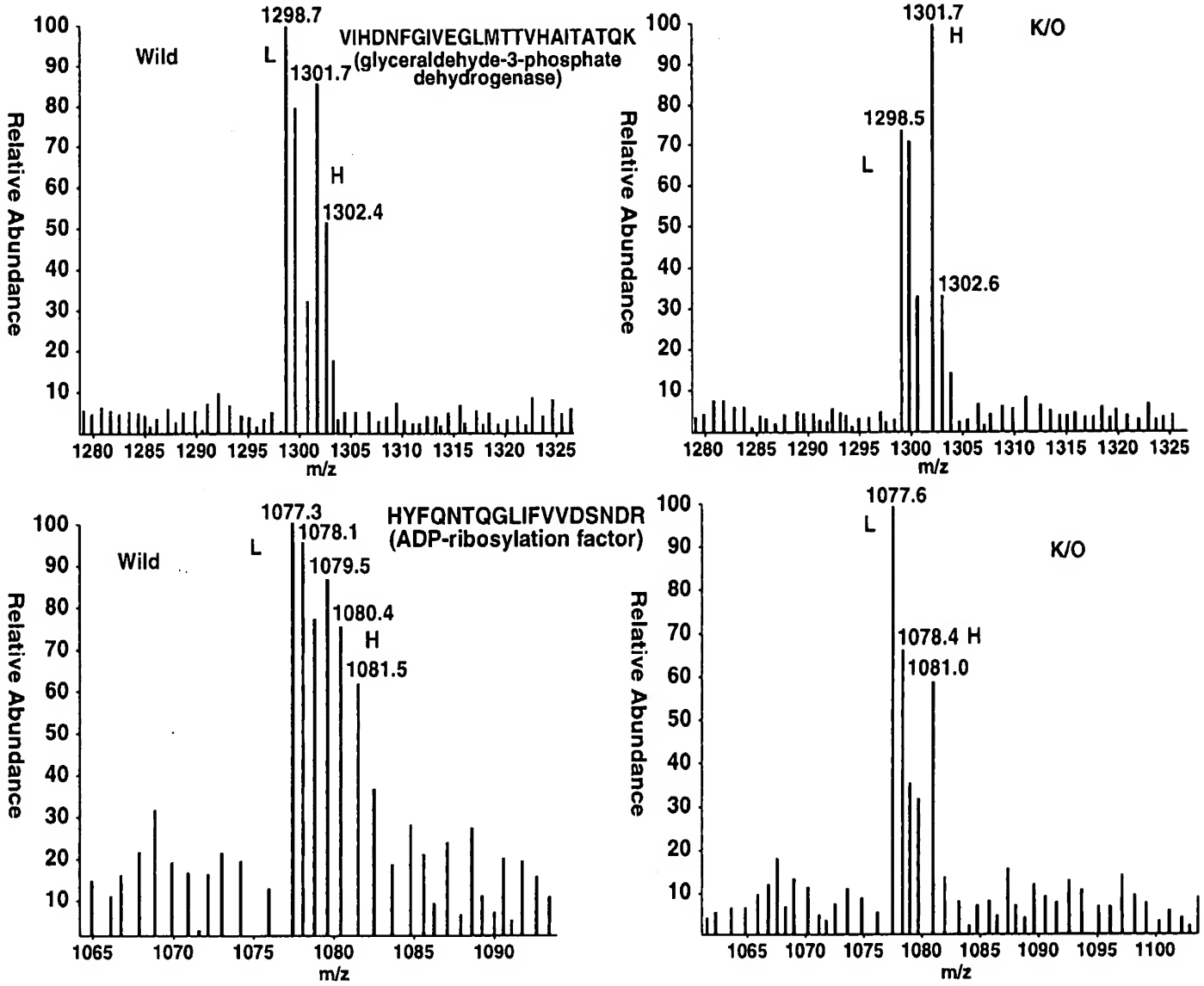
Wild
1298.7
L
1301.7
H
1302.4
VIHDNFGIVEGLMTTVHAITATQK
(glyceraldehyde-3-phosphate dehydrogenase)
Relative Abundance
m/z
K/O
1301.7
H
1298.5
L
1302.6
HYFQNTQGLIFVVDSNDR
(ADP-ribosylation factor)
1077.3
1078.1
1079.5
1080.4
1081.5
1077.6
1078.4 H
1081.0

FIG.22

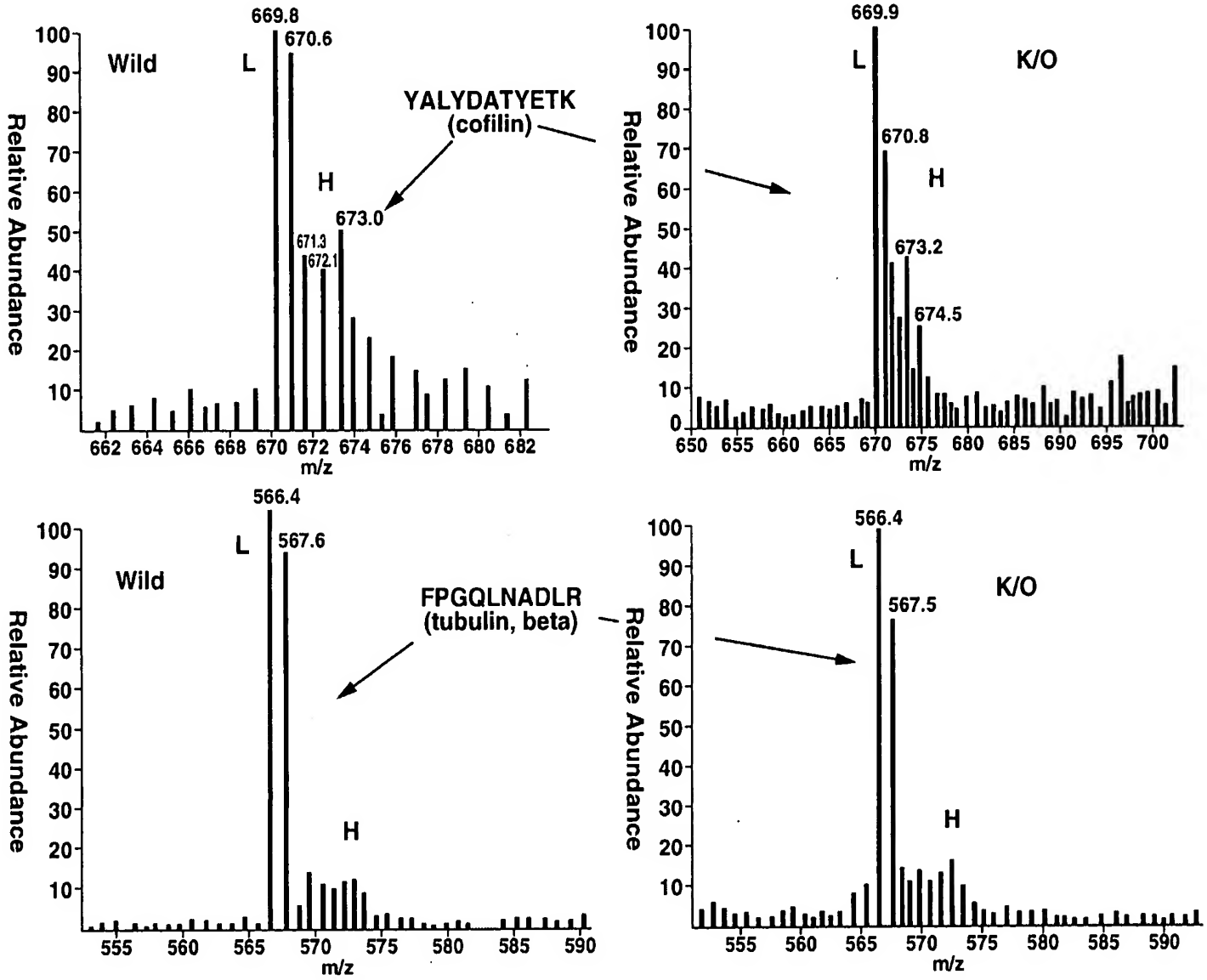
Wild
L
669.8
670.6
H
673.0
671.3
672.1
YALYDATYETK
(cofilin)
Relative Abundance
m/z
K/O
669.9
670.8
673.2
674.5
566.4
567.6
FPGQLNADLR
(tubulin, beta)
567.5

FIG.23

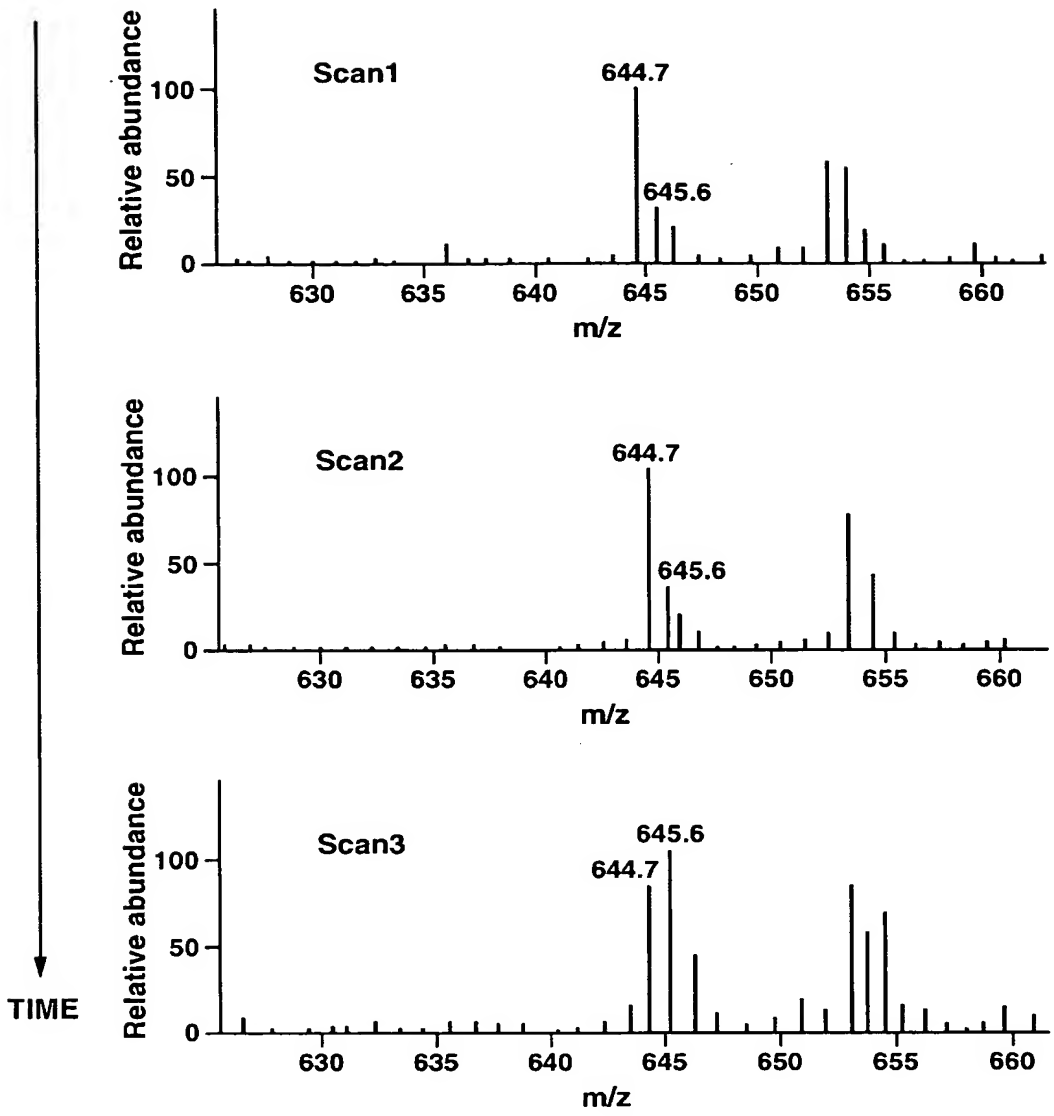
Scan1
644.7
645.6
Relative abundance
100
50
0
630
635
640
645
650
655
660
m/z
Scan2
644.7
645.6
Relative abundance
100
50
0
630
635
640
645
650
655
660
m/z
Scan3
645.6
644.7
Relative abundance
100
50
0
630
635
640
645
650
655
660
m/z
TIME

FIG.24

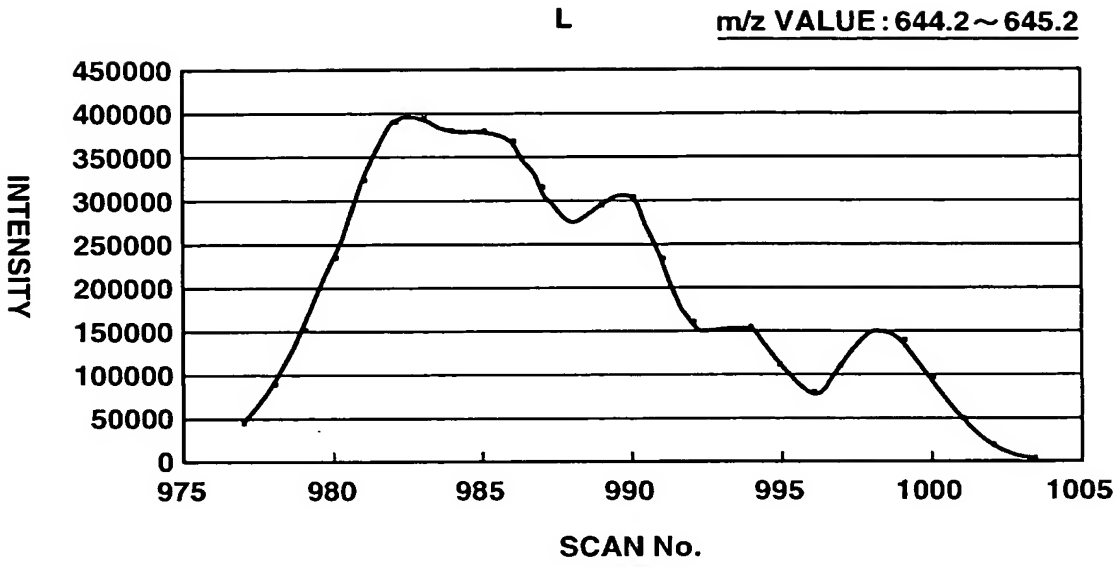
L
m/z VALUE : 644.2～645.2
INTENSITY
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
975
980
985
990
995
1000
1005
SCAN No.

FIG.25

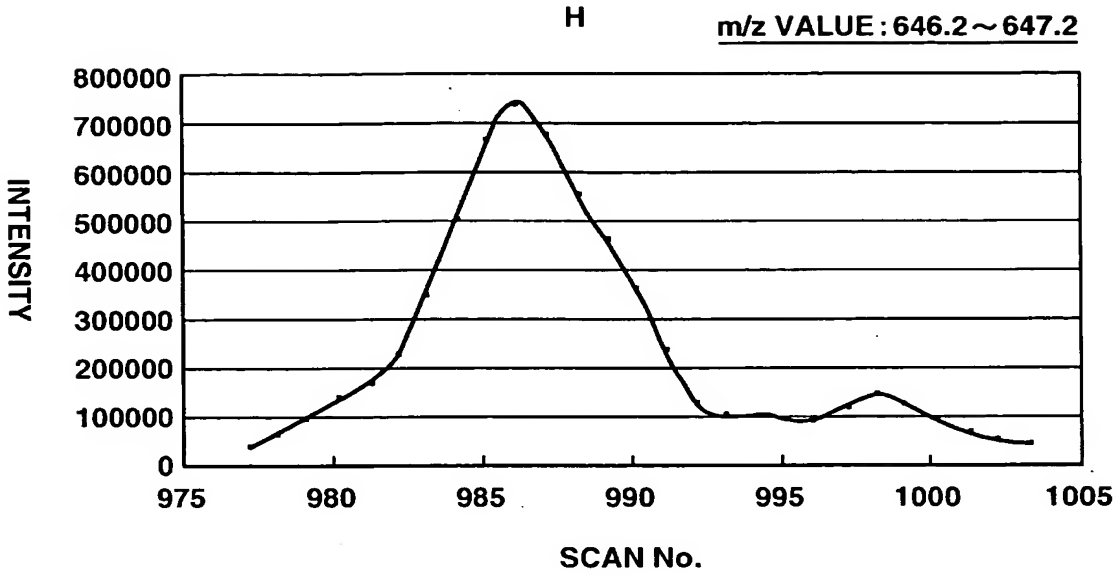
H
m/z VALUE : 646.2～647.2
800000
700000
600000
500000
400000
300000
200000
100000
0
INTENSITY
975
980
985
990
995
1000
1005
SCAN No.

FIG.26A

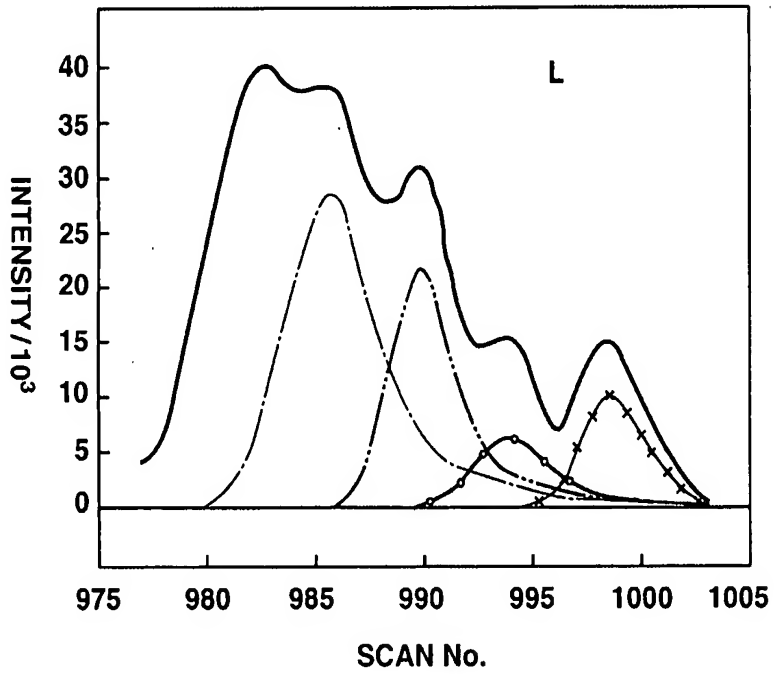

FIG.26B

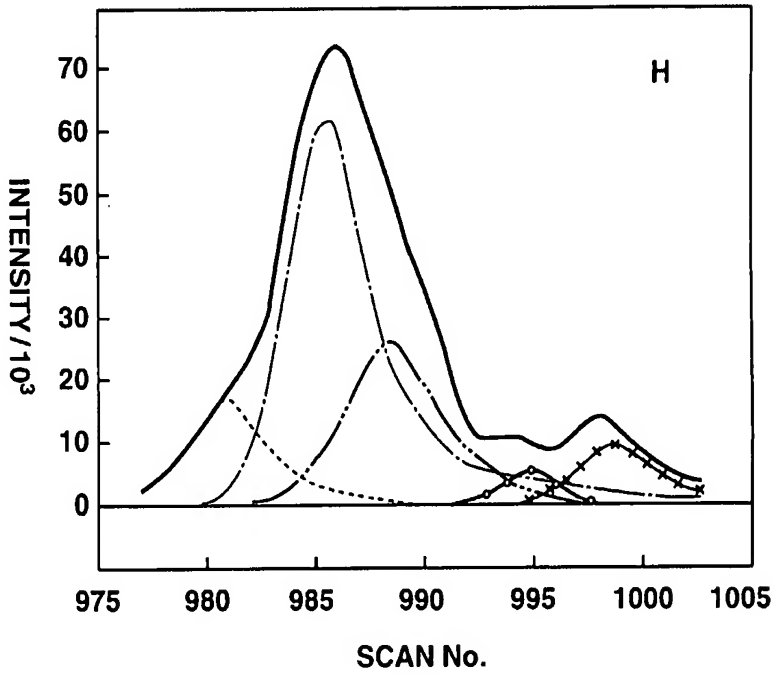

FIG.27

PEPTIDE SEQUENCE (PROTEIN NAME)	Wild PEAK INTENSITY RATIO/KNOCK OUT PEAK INTENSITY RATIO *LABELED MOLECULE WITH THE SAME SEQUENCE WAS USED AS THE INTERNAL STANDARD	Wild PEAK INTENSITY RATIO/KNOCK OUT PEAK INTENSITY RATIO **LABELED MOLECULE WITH A DIFFERENT SEQUENCE WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.1: AHQLVMEGYNWCHDR (protein phosphatase 2a) SEE FIG. 10	2.92/3.09=0.95	2.81/3.82=0.74 LABELED MOLECULE WITH THE SEQUENCE NO. 2 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.2: HHITPLLSAVYEGHVSCVK (myotrophin) SEE FIG. 10	2.85/3.97=0.72	2.96/3.21=0.92 LABELED MOLECULE WITH THE SEQUENCE NO. 1 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.3: HFSVEGQLEFR (heat shock protein 1) SEE FIG. 11	0.45/0.46=0.97	2.58/3.03=0.85 LABELED MOLECULE WITH THE SEQUENCE NO. 4 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.4: AISHEHSPSDLEAHFVPLVK (protein phosphatase PP2A) SEE FIG. 11	2.35/2.14=1.10	0.41/0.33=1.23 LABELED MOLECULE WITH THE SEQUENCE NO. 3 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.5: VIHDNFGIVEGLMTTVHAIT ATQK (glyceraldehyde-3-phosphate de hydrogenase) SEE FIG. 12	1.18/0.73=1.61	1.86/2.07=0.90 LABELED MOLECULE WITH THE SEQUENCE NO. 6 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.6: HYFQNTQNTQGLIFVVDSNDR (ADP-ribosylation factor) SEE FIG. 12	1.84/1.86=0.99	0.89/1.18=0.75 LABELED MOLECULE WITH THE SEQUENCE NO. 5 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.7: YALYDATYETK (cofilin) SEE FIG. 13	2.17/2.60=0.83	10.8/9.2=1.17 LABELED MOLECULE WITH THE SEQUENCE NO. 8 WAS USED AS THE INTERNAL STANDARD
SEQUENCE NO.8: FPGQLNADLR (tubulin, beta) SEE FIG. 13	11.38/9.4=1.21	2.28/2.66=0.86 LABELED MOLECULE WITH THE SEQUENCE NO. 7 WAS USED AS THE INTERNAL STANDARD

FIG.28

SEQUENCE	QUANTITATION NUMBER
ALVLELCCNDESGEDVEVPYVR	1.51091043
KPLLESGTLGTK	1.351344211
NFPNAIEHTLQWAR	0.379425372
NFPNAIEHTLQWAR	1.006616361
NFPNAIEHTLQWAR	0.498511448
NFPNAIEHTLQWAR	0.620944209
NFPNAIEHTLQWAR	0.744001926
NFPNAIEHTLQWAR	0.39584672
NFPNAIEHTLQWAR	0.569211695
NFPNAIEHTLQWAR	0.682017147
NFPNAIEHTLQWAR	0.362867677
NFPNAIEHTLQWAR	1.20440506
NFPNAIEHTLQWAR	1.443092102
NFPNAIEHTLQWAR	0.767798114
NFPNAIEHTLQWAR	1.736296952
NFPNAIEHTLQWAR	0.2713649
NFPNAIEHTLQWAR	2.245947181
NFPNAIEHTLQWAR	3.110330946
NFPNAIEHTLQWAR	0.251063062
YFLVGAGAIGCELLK	21.56982391
YFLVGAGAIGCELLK	0.651272581
YFLVGAGAIGCELLK	8.513072128
YFLVGAGAIGCELLK	5.037104157

FIG.29

SEARCH TARGET: ALVLELCCNDESGEDVEVPYVR

IDENTIFIED PROTEINS:

gi | 23510340 | ref | NP_695012.1 |
gi | 35830 | emb | CAA40296.1 |
gi | 24485 | emb | CAA37078.1 |

SEARCH TARGET: KPLLESGTLGTK

IDENTIFIED PROTEINS:

gi | 23510340 | ref | NP_695012.1 |
gi | 35830 | emb | CAA40296.1 |
gi | 24485 | emb | CAA37078.1 |

SEARCH TARGET: NFPNAIEHTLQWAR

IDENTIFIED PROTEINS:

gi | 23510340 | ref | NP_695012.1 |
gi | 35830 | emb | CAA40296.1 |
gi | 24485 | emb | CAA37078.1 |

SEARCH TARGET: YFLVGAGAIGCELLK

IDENTIFIED PROTEINS:

gi | 23510340 | ref | NP_695012.1 |
gi | 35830 | emb | CAA40296.1 |
gi | 24485 | emb | CAA37078.1 |

FIG.30A

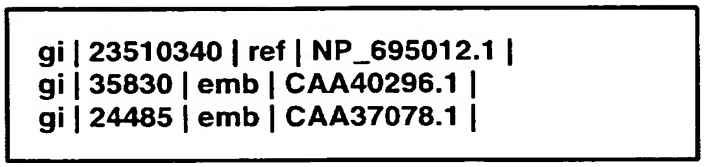
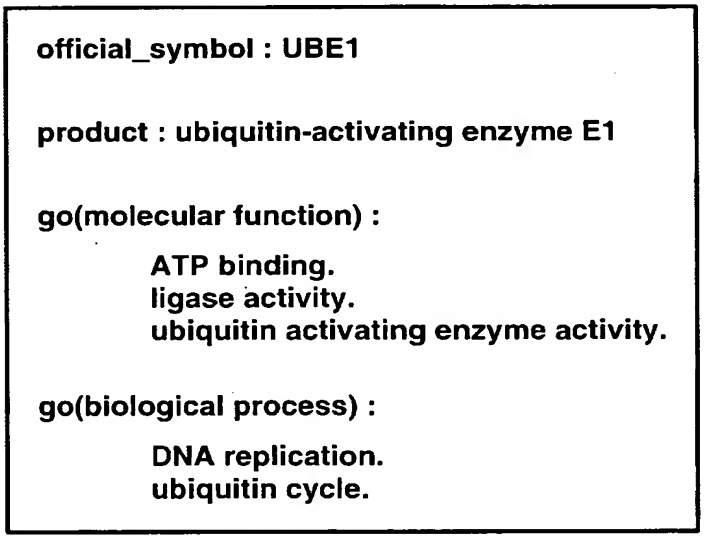
FIG.30B